REVUE

A STARFISH OOCYTE USER'S GUIDE

by

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Résumé

La maturation de l'ovocyte d'étoile de mer déclenchée in *nitro* par l'hormone naturelle, la 1-Methyladenine, est devenue un modèle d'étude de l'activation cellulaire par un agoniste spécifique externe. Cet article fait la revue des techniques originales employées dans l'étude de cette cellule en soulignant les avantages particuliers de ce matériel biologique marin. La préparation de suspensions homogènes d'ovocytes est décrite; les critères de maturation sont définis ainsi que ses trois principales caractéristiques : concentration-seuil de 1-Methyladenine, cinétique de la maturation et période hormone-dépendante. Les techniques des microinjections intracytoplasmiques et intranucléaires sont exposées en détail ainsi que les différentes manipulations auxquelles se prête l'ovocyte d'étoile de mer (élimination de l'enveloppe vitelline, stratification de l'ovocyte, préparation de fragments nucléés et anucléés, fusion d'ovocytes, colorations localisées). Les techniques d'isolement de cortex, de noyaux, de fuseaux méiotiques et de molécules spécifiques sont présentées. Enfin les conditions de la fécondation et de l'activation parthénogénétique sont décrites.

Abbreviations

ASW	Artificial sea-water
Ca, MgFASW	Calcium-, Magnesium-free artificial sea-water
cAMP	cyclic Adenosine 3', 5'-monophosphate
cGMP	cyclic Guanosine 3', 5'-monophosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EGTA	Ethylene glycol-bis (-amino-ethyl ether) N, N, N' N'-tetra acetic acid.
GSS	Gonad Stimulating Substance
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
HDP	Hormone Dependent Period
MBL	Marine Biological Laboratory (Woods Hole)
1-MeAde	1-Methyladenine
MPF	Maturation Promoting Factor
NSW	Natural Seawater
RM	Right-hand micromanipulator
RNA	Ribonucleic acid
TRIS	Tris(hydroxymethyl) aminomethane

Keg words: starfish; oocyte maturation; fertilization; methodology; intracellular microinjection; oocyte fusion; cellular fractionation.

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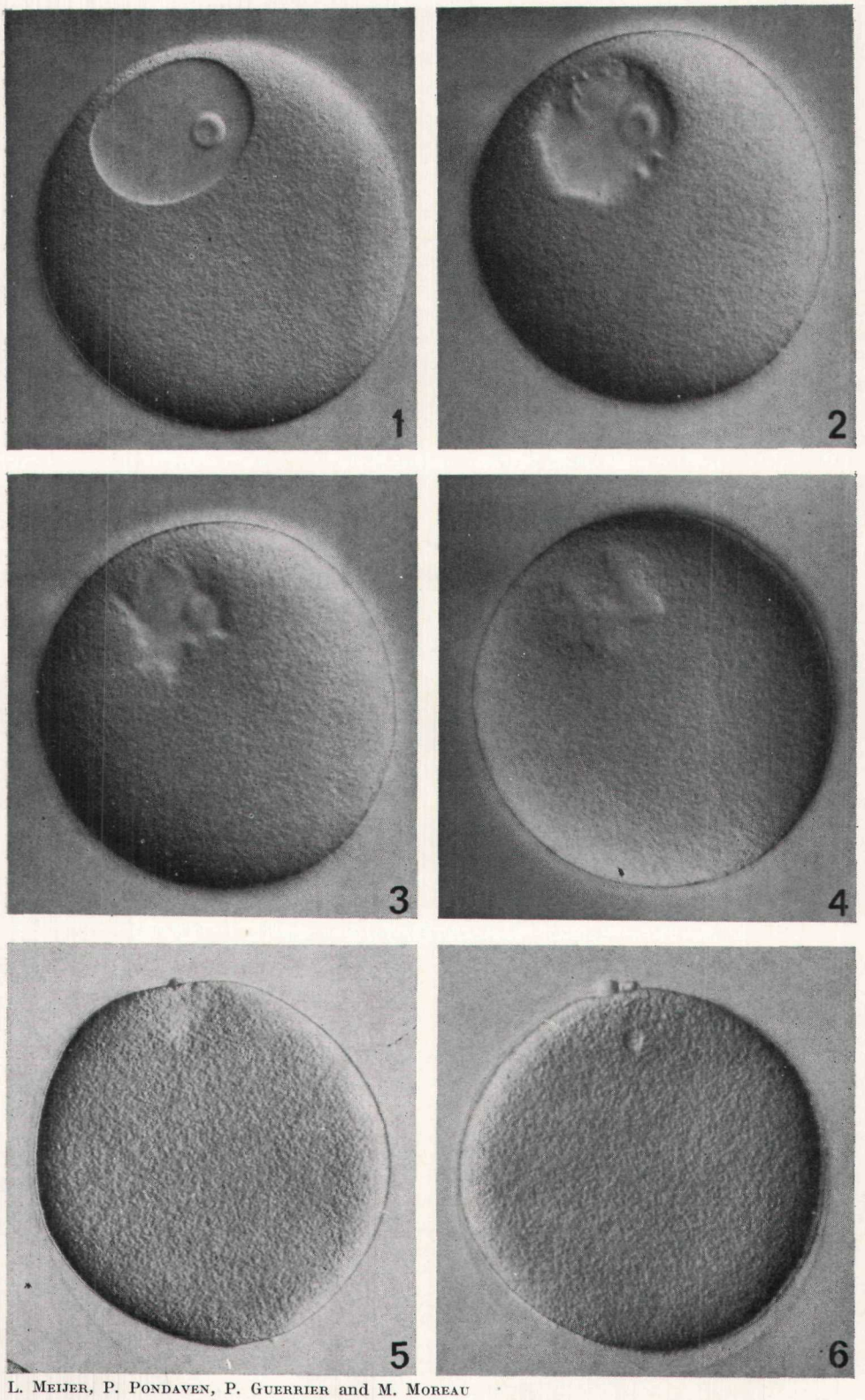
1. INTRODUCTION

At the end of oogenesis, starfish gonads are loaded with prophase-arrested oocytes, characterized by their large nucleus, or germinal vesicle (GV) (Pl. 1, Fig. 1). At the time of spawning, a hormonal system is switched on which induces the completion of meiosis (or maturation) of oocytes and the subsequent release of «ready-to-



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1 to 4. *Marthxtsteriax glacialis:* 1, intact follicle-free oocyte ;
2, 3, 4, breakdown of the germinal vesicle following 1-MeAde addition;
5, 6, *Asterias rubens:* 5, first polar body extruded, absence of nuclear reconstitution; 6, female pronucleus after extrusion of the second polar body.

be fertilized» eggs. This hormonal mechanism has been elegantly dissected by Chaet *et al,* Kanatani *et al.* and Schuetz *et al.:* a neuropeptide, the gonad-stimulating susbtance (GSS), acts on the follicle cells surrounding the oocytes to produce a second hormone, which has been identified as 1-Methyladenine (1-MeAde) by Kanatani *et al.* (1969b). This hormone then acts at the level of the plasma membrane and induces the appearance of an intracellular factor, the maturation-promoting factor (MPF), able to induce maturation when injected into unstimulated oocytes (Kishimoto and Kanatani, 1976, 1977), Finally these maturing oocytes can be fertilized by sperm and this process initiates a series of events resulting in cleavage and embryonic development. A review of the cellular mechanisms underlying both maturation and fertilization has been published recently (Meijer and Guerrier, 1984).

Since the early discovery of this hormonal system, starfish oocytes have become a new model of cellular activation because of the following reasons :

1—the abundance of starfish,

2—their usually extended spawning season,

3—the large amounts of oocytes in their gonads ($>$ 50 ml of packed cells can be prepared from a single *Marthasterias),*

4—the easy isolation of oocytes which remain arrested in the prophase stage,

5—the low requirements of oocytes which remain in perfect condition at room temperature in natural filtered sea-water (NSW) or in 5-6 components artificial sea-waters (Table 1),

6—the possibility to induce *in vitro* maturation of these isolated oocytes by the simple addition of 1-MeAde,

7—he high synchrony of these oocytes,

8—their rapid response to the hormonal stimulus (about 20 min for the first visible morphological event, i.e. the rupture of the nuclear envelope or germinal vesicle breakdown (GVBD) (Pl. 1, Figs. 2, 3, 4), 2 hours for completion of meiosis) (Pl. 1, Fig. 6),

9—the transparency of the oocytes,

10—the large size of the cells ($>$ 100 µm diameter) and of the nuclei ($>$ 50 µm), which allows easy micromanipulations such as micro-injections, preparation of nucleated and anucleated fragments, microsurgery, ... etc.,

11—their easy *in vitro* fertilization,

12—the relatively simple mechanisms of activation (independent, at least in its first steps, of DNA, RNA and protein synthesis).

For all these reasons isolated starfish oocytes provide an excellent cellular model for the study of general cellular mechanisms such as:

1—plasma mebrane receptors to an identified hormone,

2—tranduction of a hormonal message at the plasma membrane level,

TABLE 1

Composition of ASW used for starfish oocytes, according to Shapiro (1941), Van't Hoff (Cavanaugh, 1956), the Woods Hole MBL'S formulae (Cavanaugh, 1956) and Schroeder and Stricker (1983). The salt concentrations are given in g./L (mM in parentheses).

ASW	Salts (MW)	NaCl (58.44)	KCl (74.56)	$MgCl_2 6H_2 0$ (203.30)	$MgSO_4 7H_2 0$ (246.48)	$CaCl_2 2H_2 0$ (147.02)	$Na_2 SO_4$ (142.04)	$NaHCO_3$ (84.01)	EGTA (380.4)	Tris/HCl (121.14)	$H_3 BO_3$/NaOH (61.83)
SHAPIRO'S ASW		26.43 (452.20)	0.75 (10.08)	5.10 (25.09)	4.24 (17.20)	0.65 (4.43)	—	—	—	1.21 (10.00)	—
SHAPIRO'S CaFASW		26.43 (452.20)	0.75 (10.08)	6.06 (29.80)	4.24 (17.20)	—	—	—	—	1.21 (10.00)	—
VAN'T HOFF'S ASW		26.90 (460.30)	0.754 (10.11)	7.30 (35.91)	4.31 (17.49)	1.35 (9.18)	—	—	—	—	1.24 (20.06)
VAN'T HOFF'S CaFASW		27.80 (475.70)	0.754 (10.11)	7.30 (35.91)	4.31 (17.49)	—	—	—	—	—	1.24 (20.06)
MBL'S ASW		24.72 (423.00)	0.67 (9.00)	4.66 (22.94)	6.29 (25.50)	1.36 (9.27)	—	0.18 (2.15)	—	—	—
MBL'S CaFASW		25.53 (436.86)	0.67 (9.00)	4.66 (22.94)	6.29 (25.50)	—	—	0.18 (2.15)	—	—	—
MBL'S MgFASW		28.15 (481.69)	0.67 (9.00)	—	—	1.36 (9.27)	7.24 (50.97)	0.18 (2.15)	—	—	—
MBL'S Ca,MgFASW		27.00 (462.01)	0.80 (10.73)	—	—	—	1.00 (7.04)	0.18 (2.15)	—	—	—
SCHROEDER and STRICKER'S CaFASW		22.21 (380)	6.34 (8.50)	9.96 (49)	—	—	3.69 (26.00)	0.19 (2.25)	0.95 (2.50)	1.21 (10.00)	—

3—generation of a universal MPF, which apparently acts as a non-species—specific intracellular mitosis-or meiosis-inducing agent, as shown by interspecies transfer experiments (Kishimoto *et al.,* 1982),

4—hormone-induced post-translational modifications of proteins,

5—intracellular control of nuclear envelope breakdown, a general process occurring in most dividing cells,

6— interaction of sperm with eggs and the subsequent onset of synchronized cleavage.

The two aims of this paper are:

1—to provide an easy to follow guide for the utilization of starfish oocytes in Developmental Biology courses,

2—to point out the technical advantages and easiness of use of these cells and to promote their utilization in the study of general cell biology. It will therefore not provide any details on the biological system itself but will focus on the methodology and the major techniques used on starfish oocytes.

2. SOLUTIONS

2.1. Artificial sea-waters

The best culture medium for starfish oocytes is natural sea-water (NSW), which should just be freshly millipore-filterd. However in a few instances, chemically defined mediums are required, mainly calcium-free artificial sea-water (CaFASW).

The composition of the most widely used artifical sea-waters is given in Table 1. They can be prepared in stocks and kept in cold rooms for some time. Their pH should be adjusted to the pH of the local NSW. Isopycnic sea water is obtained by mixing 1 volume of NSW and 3 volumes of sucrose 0.85 M (Harvey, 1931).

2.2. 1-Methyladenine and other agonists

1-Methyladenine is prepared as a 10^{-3} M stock solution in distilled water and can be kept as such for a few years without apparent degradation or even bacterial contamination. Dithiothreitol, a disulfide-reducing agent able to induce maturation (Kishimoto and Kanatani, 1973) is best prepared freshly as a 100 mM stock solution in distilled water. Methylglyoxal-bis-(guanylhydrazone), another 1-MeAde mimetic (Meijer and Guerrier, 1983) should be prepared freshly as a 100 mM stock solution in distilled water. It irreversibly precipitates after 1-2 days. Finally, arachidonic acid, another maturation inducer (Meijer *et al.,* 1984), is prepared in 10 or 100 mM stock solutions in ethanol or dimethylsulfoxide and can be stored under nitrogen at-20°C. However it may lose some of its activity upon

exposure to air, light and room temperature. Ionophore A 23187 is prepared in 2 or 5 mM stock solution in ethanol and stored in the dark at -20°C.

3. OBTAINING AND PREPARING THE GAMETES

3.1. Preparing the oocytes

Gonads are dissected by cutting the starfish body wall along the arm. Repetitive procurement of gametes from an individual starfish is possible by using one arm at a time; spontaneous autotomy of the used arm then occurs (Fuseler, 1973). The gonad is rapidly washed in ice-cold CaFASW and delicately teared in ice-cold CaFASW with two fine forceps, in less than 1-2 minutes; the suspension is filtered through a double layer of cheese-cloth and centrifuged at low speed for 0.5-1 min; follicle cells remain in the supernatant and are eliminated. The pelleted oocytes are gently resuspended in ice-cold CaFASW and recentrifuged. This washing is repeated until no more follicle cells remain and a homogenous population of GV-arrested oocytes is obtained.

The use of CaFASW in preparing the oocytes is important since the presence of calcium induces the release of 1-MeAde by follicle cells and the absence of calcium facilitates the detachment of these cells from the oocyte surface. The cold temperature increases the necessary contact time for 1-MeAde lo induce maturation (see 4.5).

The prepared oocytes are best maintained under slow and constant stirring in large volumes of sea water or CaFASW. They can be used for several hours up to a day. Before an experiment is performed, the percentage «spontaneous maturation» should be determined. Finally the oocyte concentration can be adjusted to specific values after two types of estimation :

—Volume oocytes volume of suspension: A 1 ml sample of suspension is centrifuged for 2-3 min. in a graduated centrifuge tube. The concentration is expressed as per cent (v/v): a 10 per cent suspension contains 100 µl packed oocytes/1000 µl suspension.

— Number of oocytes/volume of suspension: the number of oocytes is counted in a small volume (10 µl of suspension or of a dilution of it) either directly or in a capillary tube. The concentration is expressed in number of oocytes/ml of suspension.

The oocytes suspension concentration is best adjusted to a definite value before experimentation.

3.2. Preparing the sperm

Sperm is obtained by tearing the isolated male gonad without medium and keeping it «dry» until use, at a cold temperature (refri-

gerator). It is diluted before fertilization at specific concentrations, using a calibration curve (the O.D. at 460 nm is proportional to the sperm density) (Rothschild, 1950; Vacquier and Payne, 1973).

4. INDUCING OOCYTE MATURATION

4.1. Criterions for maturation

The first clearcut sign of maturation is the rupture of the nuclear envelope (Pl. 1, Figs. 2, 3, 4) or germinal vesicle breakdown (GVBD) : it occurs within 20 minutes following the start of hormonal stimulation (Fig. 1B). Although it has been widely used as the criterion for maturation, meiosis is only completed after the emission of the two polar bodies and the appearance of the female pronucleus, which are only terminated about 2 hours after hormone addition (PI. 1, Figs. 5, 6)..

Furthermore, maturation, *sensu stricto,* can only be ascertained by a normal cleavage and embryonic development after successful fertilization. Despite these ambiguities we believe that GVBD is a good criterion for the initiation of maturation. Recent studies have indeed shown that the simple mechanical breakdown of the nuclear envelope by itself is sufficient to induce the completion of meiosis and to lead to perfectly fertilizable eggs (Guerrier *et al.,* 1983).

4.2. The oocyte maturation assay

Our oocyte maturation assays are usually performed in plastic plates with 1.5 ml wells. Typically the following components are added successively:

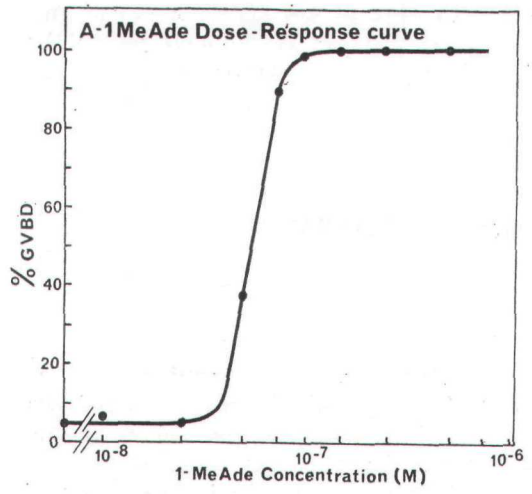
(900 - x - y) µl NSW or CaFASW
100 µl oocyte suspension (10 p. 100)
x µl inhibitor or other antagonist
y µl 1 MeAde or other agonist

in a total 1000 µl final volume. The *Marthasterias* and *Asterias* oocytes are able to withstand up to 10 µl dimethylsulf oxide or ethanol/ 1000 µl, as long as they are rapidly stirred upon addition of these organic solvents. They are also able to mature in the presence of up to 100 µl distilled water/1000 µl.

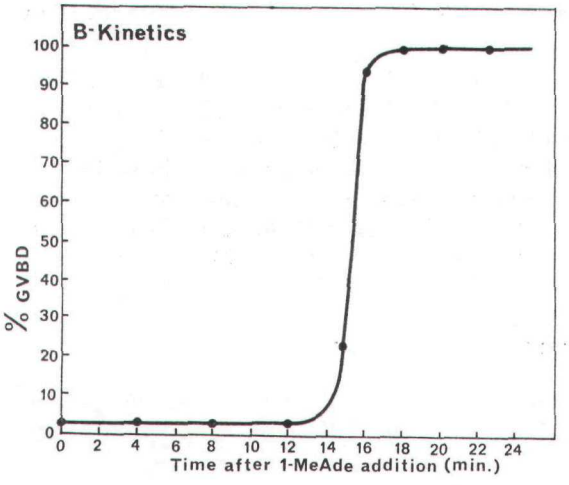
Maturation is checked after 30 min and the percentage GVBD is recorded after counting at least 200 oocytes under the microscope.

Three criterions characterize an oocyte batch and should be determined during a series of experiments:
—the 1-MeAde threshold concentration,
—the kinetics of maturation,
—the hormone-dependent period,

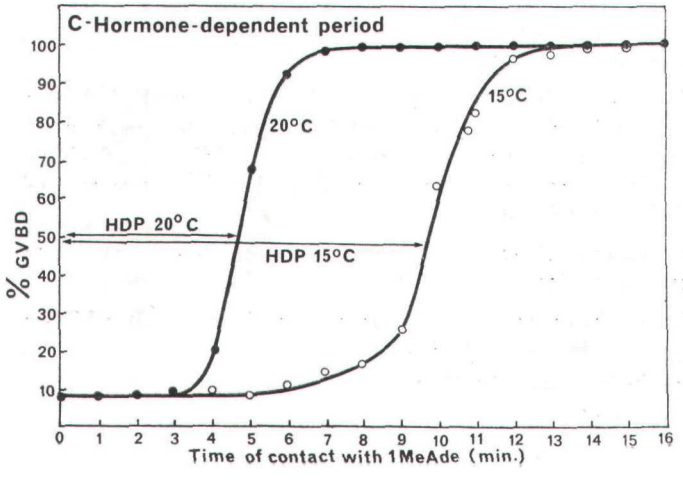
FIG. 1



A. 1-MeAde dose-response curve



B. Kinetics of 1MeAde-induced GVBD



C. Determination of the hormone-dependent period at two temperatures.

4.3. The 1-Merhyladenine threshold concentration (Fig. 1A)

Before starting an experiment with an oocyte batch, its 1-MeAde threshold concentration should be determined. This is particularly important for the studies involving the use of inhibitors whose action is generally reversed by high concentrations or 1-Me-Ade. The threshold concentration is determined in a series of wells in which various concentrations or 1-MeAde are added (from stock solutions of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}M). It lies around 10^{-7}M, but varies between 2.5 X 10^{-8}M and 2.5 X 10^{-7}M, according to the species, the time in the spawning period, the oocyte batch, the time after oocytes preparation. The threshold concentration does not depend on temperature nor on time of contact of 1-MeAde with the oocytes.

4.4. The kinetics of maturation (Fig. 1B)

Kinetics of GVBD are obtained by recording the percentage GVBD of aliquots as a function of time after addition of 1-MeAde to an oocyte batch. Since it depends on temperature, it is more accurately determined when the oocytes are incubated in a constant temperature water bath (20°C, for ex.). However kinetics are independent of 1-MeAde concentration.

4.5. The hormone-dependent period (Fig. 1C)

The hormone-dependent period (HDP) is the period of time during which the presence of 1-MeAde is required for induction of maturation. If 1-MeAde is removed (by washing the oocytes) during this period the oocytes do not mature; if 1-MeAde is removed after the end of this period, maturation still occurs. This period can thus be viewed as a period of reversibility, followed by a period where maturation is irreversibly induced. Repeated short treatments with 1-MeAde can be cumulated to reach the length of the HDP.

The HDP is determined as follows : at time O, a treshold concentration of 1-MeAde is added to a 10 ml suspension of oocyte. At various times 200 µl aliquots are injected in tubes containing 12 ml NSW so that the 1-MeAde concentration reached falls below the threshold value. After 30 min the percentage GVBD is recorded in the various oocyte aliquots which have been arrested when diluted during the HDP but not when diluted afterwards. An alternative to the dilution method for other agonists, is the use of specific «chelators», such as bovine serum albumin for arachidonic acid. The HDP is independent of the 1-MeAde concentration but is highly dependent on temperature and its determination should therefore be performed at constant temperature,

5. INTRACELLULAR MICROINJECTIONS

5.1. Microinjecfion set-up

Intracellular microinjections are performed according to the method of Hiramoto (1974), Kishimoto and Kanatani (1976): a constricted and oil-filled micropipette is impaled into an oocyte immobilized under the microscope and pressure-injection is performed with a screw-microinjector syringe.

The set-up is composed of:
—a stable working surface,
—a microscope,
—a right-hand micromanipulator holding the micropipette connected to,
—a screw-syringe microinjector,
—a left-hand micromanipulator holding the «constriction system» connected to,
—a small power supply.

1) *The working surface:* the bearing surface for the set-up should be a plate of iron resting on large rubber stoppers on a stable table.

2) *The microscope:* any microscope with the following features can be used:
—a stable stage, which does not move up and down during focussing (this is an absolute requirement!),
—a 10 × ocular with a micrometer,
—a set of objectives : 10X, 20X and 40X.

3) *The right hand micromanipulator* is preferably fastened with a magnetic base to the iron plate or to an iron intermediate stand (according to the height of the microscope stage). A hub for micropipettes is fastened to it and the micromanipulator is positioned so that the micropipette will arrive perpendicularly to the right side of the microscope stage. On one side of the hub a micropipette will be inserted, on the other side a piece of tubing will be connected to the microinjector.

4) *The microinjector* should be a screw-syringe (0.5 mm/rotation) fixed to the iron plate by a magnetic base, at some distance of the micromanipulator.

5) *The left-hand micromanipulator* is only used to perform the constrictions during the preparation of micropipettes and so is better removed out of the way when microinjections are performed. This micromanipulator provides the stand of a holder at the extremity of which a platinum wire loop is fixed which induces the microelectrode constriction upon heating. This platinum wire is electrically connected to a simple power supply.

6) *The power supply* provides a 0 to 6 volts (2-5 A) DC current to the platinum wire (0.1 mm diameter),

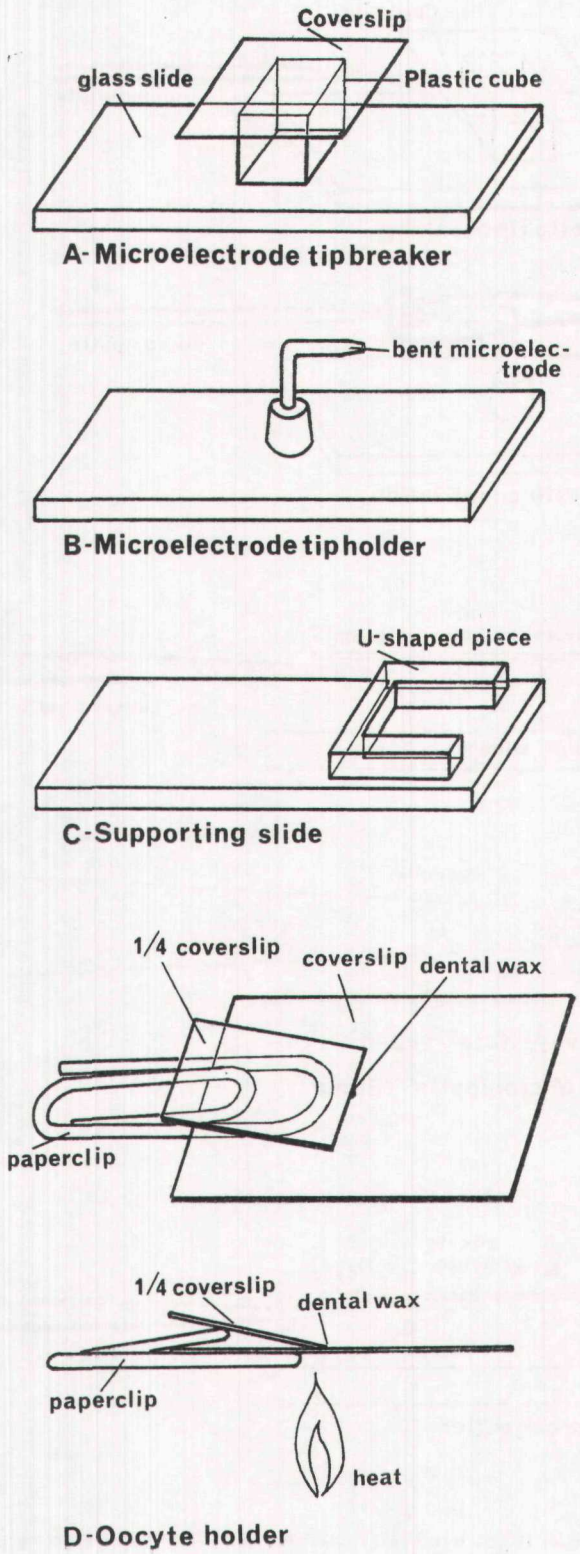


FIG. 2
Microinjection accessories

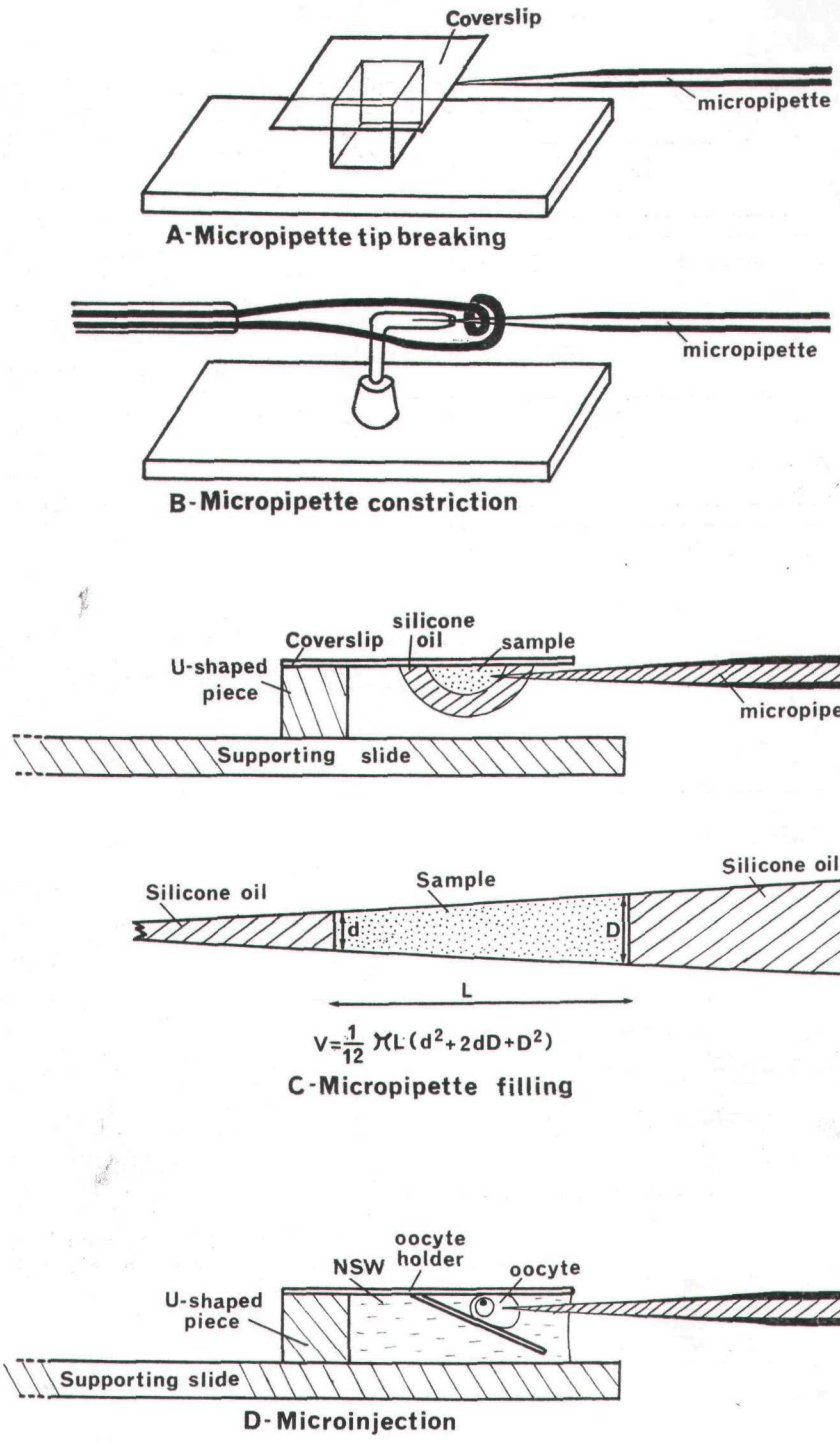


FIG. 3

Micropipette preparation (RM, Right hand micromanipulator and microinjection

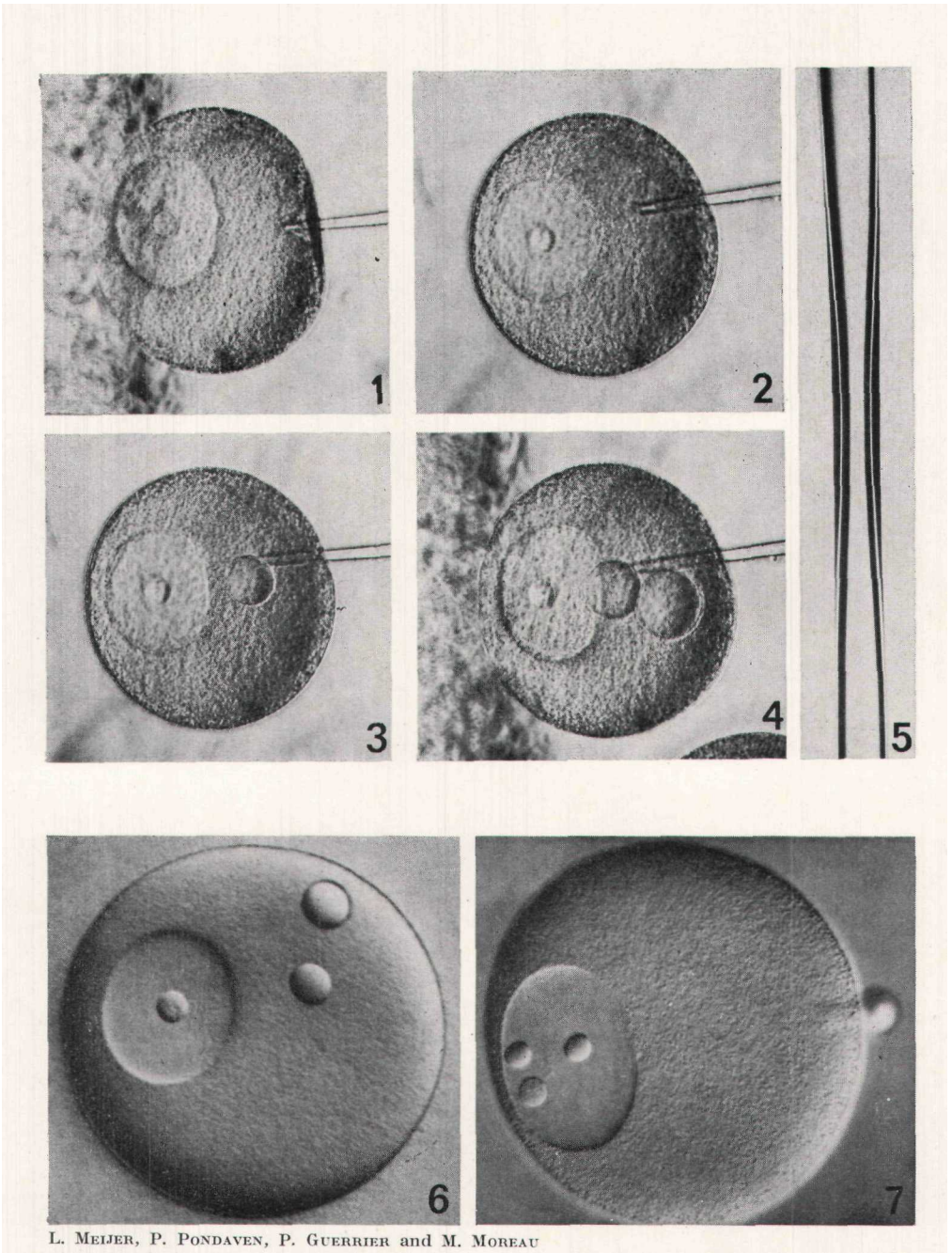


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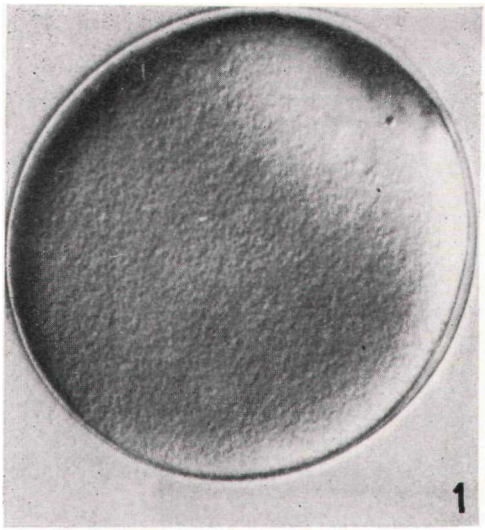
PLATE 2

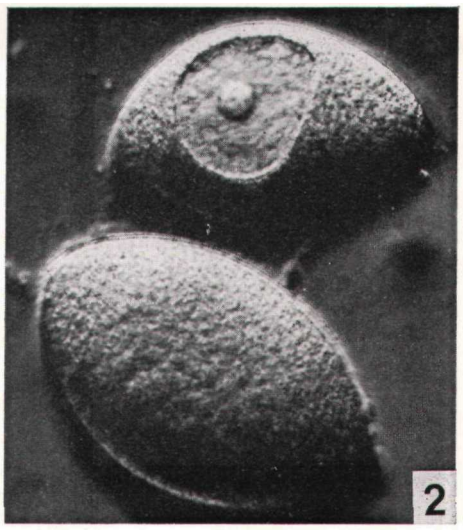
1 to 4. *Marthasterias glacialis:* successive steps in microinjection of a sample included between two oil drops.

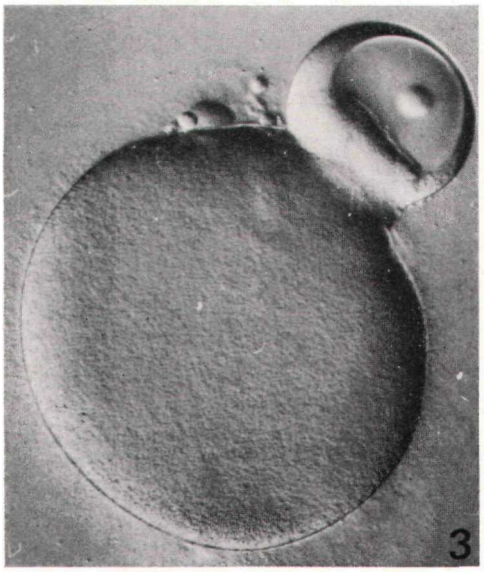
5. Constriction of the micropipette.

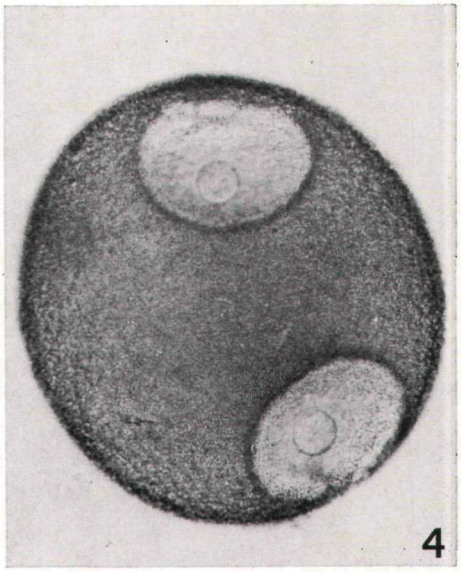
6. *Astropecten auranciacus:* intracytoplasmic microinjection.

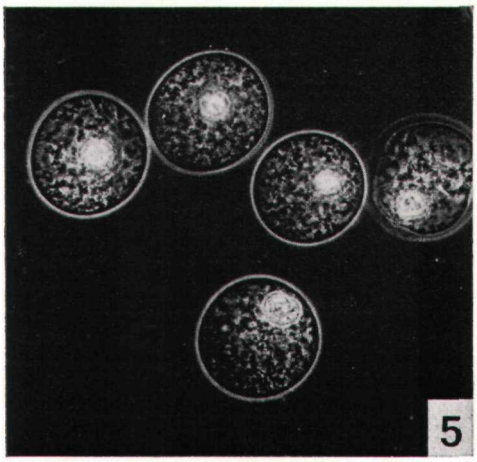
7. *Marthasterias glacialis:* intranuclear microinjection. The bleb marks insertion point of the micropipette.











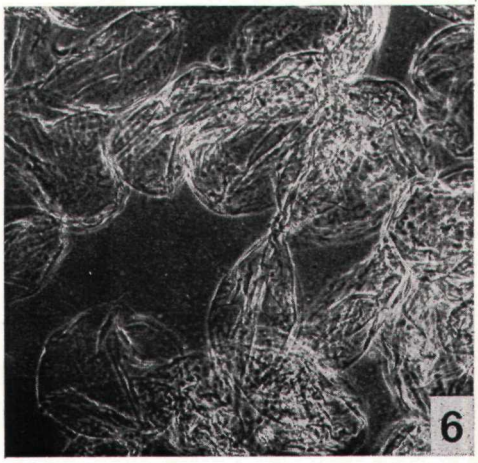


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PLATE 3

5.2. The accessories (Fig. 2)

Four simple accessories are required for preparing the micropipettes (the «tip breaker» and th «micropipette tip holder») and for microinjecting (the «supporting slide» and the «oocyte holder»). They all should be prepared in advance.

1) *the «tip-breaker»* (Fig. 2A) consists of a coverslip glued with dental wax on top of a piece of plastic itself glued on a slide.

2) *the «micropipette tip holder»* (Fig. 2B) is made of a bent (90°) micropipette broken at its end and fixed vertically to a cylindrical holder (provided with the Drummont «microcaps») glued on a glass slide.

3) *the «supporting slide»* (Fig. 2C) consists of a U-shaped piece of plastic glued on a glass side.

4) *the «oocyte holder»* (Fig. 2D) consists of a coverslip to which a piece (1/4) of a coverslip is glued at a slight angle. These holders are easily made with a wire paper-clip: coverslips are broken into 4 quarters with a diamond. Then a paper-clip is used to hold the coverslip piece on a coverslip at a slight angle. A tiny piece of dental wax is put at the junction of both pieces of glass and rapidly melted over a small flame. After cooling the paper-clip can be removed, leaving an «oocyte holder».

5.3. Preparing the micropipettes (Fig. 3)

A whole set of micropipettes is prepared before a series of microinjections. The micropipette preparation can be divided in four stages:

— micropipette pulling,
— mcropipette tip breaking,
— micropipette constricting,
— micropipette filling.

1) *microelectrode pulling:* 50 µl capillary glass tubes (Drummond «microcap») are pulled in a classical microelectrode puller set up to produce regular and long-size tips (about 15 mm length).

PLATE 3

1. *Astropecten auranciacus* matured oocyte stratified by centrifugation over 23 p. 100 Ficoll in sea-water ;
2. *Marthasterias glacialis* oocyte cut in two halves with a glass needle.
3. *Astropecten auranciacus* oocyte centrifuged on a discontinuous sucrose gradient in the process of budding off a nucleated fragment.
4. Two *Marthasterias glacialis* oocytes fused at the germinal vesicle stage using polyethylene glycol.
5. *Marthasterias glacialis* isolated germinal vesicles.
G. *Marthaslerias glacialis* cortices prepared with $MgCl_2$.

2) *micropipette tip breaking* (Fig. 3A) : to get an easy impalement into the oocyte, the tip of the microelectrode is broken under the microscope against the edge of a coverslip (on the «coverslip holder» described in 5.2.1.), according to the following steps:

—set the «coverslip holder» on the microscope stage and set it in center of the field,

—bring the tip of micropipette near the edge of the coverslip,

—bring the tip in focus with the vertical movement of the micromanipulator,

—hit the micropipette tip against the corverslip edge, until the tip breaks,

—keep the micropipettes with a 2-6 µm diameter tip.

3) *micropipette constriction* (Figs. 3B and Pl. 2, Fig. 5) : micropipettes are constricted to reduce the flow during pressure-injection. The constriction is produced by a local heating of a platinum wire set around the micropipette, according to the following steps:

—set the «micropipette tip holder» on the microscope stage oriented towards the right side of the microscope,

—focus on the lateral sides of the holder and set it on the side of the visual field,

—bring the platinum loop around the «micropipette tip holder».

—bring the micropipette near the opening of the «micropipette tip holder»,

—bring it in focus with the vertical movement of the right micromanipulator,

—enter the holder with the micropipette (for about 5 mm) with the horizontal movement of the right micromanipulator,

—shift the platinum loop around the micropipette and in the microscope field,

— start heating gradually on and observe the constriction under the microscope.

4) *micropipette filling* (Fig. 3C) is performed just before microinjection. First the micropipette filled with silicone oil (DC 200) using a spinal syringe. Then it is filled with the sample to microinject according to the following steps:

—a small drop of sample is put on the center of coverslip and immediately surrounded and covered with oil,

—the coverslip is then inverted and put on a «supporting slide» (5.2.3.),

—the «supporting slide» is put on the microscope stage and focussed at the limit between the sample and the oil,

—the micropipette tip, filled with oil, is focussed at the same height with its supporting micromanipulator and is brought, through the oil, into the sample fluid,

—a small volume is aspirated with the screw-syringe microinjector,

—the micropipette tip is then shifted back into the oil and a small volume of oil is further aspirated,

—the length, small diameter and large diameter of the sample are measured and are used to calculate the volume of microinjected sample, according to the formulae given in Fig. 3C.

5.4. Intracytoplasmic microinjection (Fig. 3D and PI. 2, Figs. 2-4, 6)

First, oocytes in NSW (or preferably equilibrated in 80 p. 100 ASW) (Kishimoto and Kanatani, 1977) are inserted into an «oocyte holder» set on a «supporting slide» with NSW. Then the micropipette is filled according to 5.3.4. with a sample volume of 5-10 p. 100 the oocyte volume. The oocyte preparation is set on the microscope stage, focussed on the edge of an oocyte and shifted out of the visual field; the micropipette tip is brought in the center of the visual field and focussed with its micromanipulator vertical movement. The oocyte preparation is slowly brought back into the visual field until the micropipette tip comes close to the edge of the oocyte. It is then slowly inserted and, when in the oocyte, injection is performed slowly with the screw-syringe microinjector: first an oil droplet, then the sample and finally a second oil droplet. The oocyte preparation is then removed and the sample supporting slide is put back on the microscope stage for a refilling of the micropipette and another microinjection.

5.5. Intranuclear microinjection (Fig. 3D and PI. 2, Fig. 7)

For intranuclear microinjection, a smaller volume of sample is of course required (5-10 p. 100 of the germinal vesicle volume).

Intranuclear microinjections are best performed on oocytes oriented so that the germinal vesicle lays opposite to the site of entrance of the micropipette. Microinjection is performed at first as for intracytoplasmic microinjection: focus on the edge of the oocyte an don the tip of the pipette. When inside the oocyte, the edge of the germinal vesicle and the micropipette tip are put in focus and the micropipette can be pushed into the nucleus.

6. OOCYTE MANIPULATIONS

6.1. Removal of the jelly coat

In some species the jelly coat is removed by washing the oocytes with sea water acidified to pH 4.5 with HCl (Nemoto *et al.,* 1980) or 5.5 (Shirai and Kanatani, 1982). The observation of eggs in the presence of India ink shows the disappearance of the jelly. This method does not seem to be efficient for all species (Schroeder and Stricker, 1983); however the jelly coat is removed along with the vitelline envelope during enzymatic digestion of the oocyte investing coats (6.2.).

6.2. Removal of the vitelline envelope

The vitelline envelope can be removed either mechanically after ionophore A 23187-induced membrane elevation (Shida and Hirai, 1978) or enzymatically by a brief and microscope-controled treatment with pronase 0.05-0.1 p. 100 or trypsin 0.05 p. 100 (W/V) eventually followed by 1 M urea (Nemoto *et al.*, 1980). Alternatively a pronase 0.1 p. 100 or protease 1 p. 100 treatment after 10 µM ionophore stimulation is efficient in removing the vitelline envelope (Schroeder and StriCker, 1983). The response of these denuded oocytes to 1-MeAde remains identical to the response of vitelline envelope-bearing oocytes as far as kinetics of maturation, duration of the HDP and sensivity to 1-MeAde are concerned. They are however more sensitive to mechanical damage. Finally a brief treatment (3-5 min) with 10 mM Dithiothreitol (DTT) in NSW or CaFASW at pH 9.0-9.5 removes the vitelline enveloppe and the jelly coat (Bryan and Sato, 1970) but it should be remembered that this product induces meiotic maturation (Kishimoto and Kanatani, 1973).

6.3. Oocyte stratification (PL 3, Fig. 1)

The stratification of oocyte components in an oil cap, a hyaline layer and a pigmented layer is easily performed by centrifugation (Kishimoto *et al.*, 1977). One volume of sea water is layered on top of one volume of a 23 p. 100 Ficoll 400 solution in sea-water (W/V). The oocytes are layered at the interface and a 15 mn centrifugation at 12000* g is performed at 0°C.

6.4. Preparation of nucleated and anucleated oocyte fragments (Pl. 3, Figs. 2, 3)

Nucleated and anucleated oocyte fragments can be prepared by bissection of a vitelline coat deprived oocyte with a glass needle (Yamamoto and Yoneda, 1983; Guerrier, unpubl.). The oocyte can also be manually anucleated by squeezing the GV out from the oocyte through a perforation made with a fine glass needle in the oocyte surface near the GV (Hirai *et al.*, 1971).

However batch preparations of nucleated and anucleated oocyte fragments can also be made by centrifugation of cytochalasin B-treated cells, as first described by Nemoto *et al.* (1980): the oocytes are treated with cytochalasin B (10 µg/ml) for 10 min. and then centrifuged at 14000 g for 20 min. in the presence of cytochalasin B (3 µg/ml). Oishi and Shimada (1983) further improved the method as follows: after a 12 min. treatment with cytochalasin B (10µg/ml) the oocytes are layered on top of a discontinuous gradient of mixtures of (1) 0.85 M sucrose, 53 mM $MgCl_2$ and (2) NSW containing 2.5 µg cytochalasin B/ml: a cushion of 0.7 M sucrose, 53 mM $MgCl_2$ is overlaid by layers of 75 p. 100, 71 p. 100, 50 p. 100 and 42 p. 100

mixtures of (1) and (2). After centrif ugation at 14000 g for 20 minutes the nucleated fragments are recovered between the 42 p. 100 and 50 p. 100 layers and the anucleated fragments between the 75 p. 100 layer and the cushion. Vassetsky *et al.* (1984) followed another method: the oocytes are incubated for 30-45 min in 5 p. 100 Ficoll containing 5 µg cytochalasin B/ml and then layered on top of a discontinuous gradient of 4.5 ml 22 p. 100 Ficoll and 6.6 ml 17 p. 100 Ficoll (containing 5 ag cytochalasin B/ml); the volume is adjusted to 35 ml with ASW and the tubes are centrifuged for 25 min at 21000 g. The method of Harvey (1936), not requiring the presence of cytochalasin B, has also been applied to starfish oocytes (Doree, 1981): oocytes are simply layered on top of a discontinuous sucrose gradient (upper layer: 1/3 NSW+2/3 0.95 M sucrose; lower layer: 0.95 M sucrose) and centrifuged at 11000 g for 10 min.

6.5 Oocyte fusion (Pl. 3, Fig. 4)

A procedure for fusing starfish oocytes has been described by Sekirina *et al.* (1983) and Vassetzky *et al.* (1983, 1984). The oocyte vitelline envelope is first removed by a 15-25 min treatment with 0.25 p. 100 trypsin; the oocytes are exposed to 1 M urea, 1 mM $CaCl_2$ for 30 sec to enhance adhesion (Bennett and Mazia, 1981); a small volume of a 4-6 p. 100 oocyte suspension is then layered on top of an identical volume of 50 p. 100 (w/v) polyethylene glycol 6000 in Ca^{++}, Mg^{++} free-ASW and allowed to settle down; after 1 min the suspension is gently diluted with ASW. Up to 5-6 p. 100 fusion occurs (Sekirina *et al.,* 1983) and these hybrids undergo maturation upon addition of 1-MeAde and undergo cleavage upon fertilization (Vassetzky *et al.,* 1983). Hybrids can also be obtained between anucleated and nucleated fragments (Vassetzky *et al,* 1983).

Another method, electric field-induced fusion, has been used successfully for sea urchin eggs (Richter *et al.,* 1981; Zimmerman, 1982). It will probably be efficient for starfish oocytes provided they can withstand Ca^{++} and Mg^{++} deprivation since this method requires the use of a non-ionic medium.

6.6. Local staining of the oocyte surface

Two methods have been designed to stain the oocyte locally (Shirai and Kanatani, 1980). Oocytes can be stained in batch as follows : oocytes are layered on a small piece of cellophane sheet, the excess ASW is removed by blotting with filter paper and the piece of cellophane is set on an agar-gel plate (50 p. 100 ASW, 3 p. 100 agar, 1 p. 100 Nile Blue) prepared at the bottom of a small petri dish. The dish is covered to prevent evaporation and staining is performed for 15 min. Individual staining is performed on an oocyte sucked up into the tip of a capillary (narrower than the oocyte diameter) mounted on a micromanipulator and connected to screw-syringe. The oocyte can be set with its GV located distally, centrally or proximally to the capillary tip. The tip is then transfered to a

droplet of 0.01 p. 100 Nile Blue or 0.05 p. 100 Neutral red in ASW for local staining during a few minutes. An individual oocyte can thus be stained at two different areas with two different stains.

7. ISOLATION OF SUB-CELLULAR FRACTIONS AND PURIFICATION OF SPECIFIC MOLECULES

7.1. Isolation of cortices (Pl. 3, Fig. 6)

The oocyte cortex consists of the vitelline envelope, the plasma membrane and the 2-5 µm superficial layer of the oocyte containing the cortical granules. It can be isolated by a method described by Guerrier (1972) : oocytes are rapidly washed with 0.53M NaCl, 0.05 M Tris-Maleate pH 8.2 and then submited to 10 strokes of a hand homogeniser fitted with a teflon pestle in ice-cold buffer. After a brief (1 min) centrifugation at 1000 g a pellet is obtained which is washed several times with the buffer. Another method consists of homogenizing in $MgCl_2$ 0.1 M and washing with 0.01 M $MgCl_2$ (Sakai, 1968). A plasma membrane-enriched fraction is obtained by treating the isolated cortices for 30 min with 0.2 p. 100 Triton X 100 in 0.53 M NaCl, 0.05 M Tris-Maleate pH 8.2. After 5 min centrifugation at 1000 g a supernatant is obtained which is enriched in plasma membrane vesicles (Doree *et al.,* 1978).

7.2. Isolation of germinal vesicles (Pl. 3, Fig. 5)

Isolation of germinal vesicles is performed according to Thaller *et al.* (1969), on oocytes whose vitelline envelope has been removed (see 6.1). One volume of eggs is added to 9 volumes of 1 M sucrose, 2 mM $MgCl_2$, 2 p. 100 Triton X 100. The suspension is manually agitated and observed under the microscope. As soon as the germinal vesicles start to come out of the oocytes, the suspension is centrifuged at low speed and the germinal vesicles are washed several times with 1 M sucrose, 2 mM $MgCl_2$.

7.3. Isolation of meiotic spindles

The method employed, based on the techniques used by Mazia *et al.* (1961) and Kane (1965) on sea urchin eggs, has been applied to isolate mitotic spindles of starfish oocytes (Bryan and Sato, 1970). At fisrt the vitelline enveloppe and the jelly layer are removed using DDT at high pH (see 6.2). The oocytes are then washed twice with 1M dextrose: Ca FASW (9: 1) and resuspended in 1 volume of 12 p. 100 hexylene glycol buffered to pH 6.3 with 10 mM potassium phosphate. Vortexing disrupts the oocytes. The suspension is rapidly cooled at 0°C and centrifuged at 1000 g for 5 min; the pellet of

spindles is washed a few times with hexylene glycol. The spindles can also be isolated using 1 M sucrose, 1 mMEDTA, 0.15 M dithiodiglycol adjusted to pH 6.2 with NaOH, instead of hexylene glycol.

7.4. Purification of specific molecules

The various molecules that have been purified from starfish oocytes are listed in Table II.

TABLE 2

Molecules that have been identified in **and/or** purified **from starfish oocytes.**

Molecules	References
— Histones	VANHOUTTE-DURANT *et al.* (1977) MARTINAGE *et al.* (1985)
— Myosin	MABUCHI (1970)
— calmodulin	DOREE (1980) MEIJER and WALLACE (1980) MEIJER and GUERRIER (1981)
— *calmodulin-binding proteins*	MEIJER and WALLACE (1985)
— DNA endonuclease	CARESTIA *et al.* (1985)
— DNA ligase	OISHI and SHIMADA (1984)
— DNA polymerases	HARAGUCHI and NAGANO (1983)
— phospholipids	MEIJER *et al.* (1985)
— Arachidonic acid metabolites	MEIJER *et al.* (1985)
— Asterosaponin	VOOGT and VAN RHEENEN (1979)
— polyamines	MEIJER and GUERRIER (1984) MEIJER *et al.*, unpubl.
— adenine nucleotides	SCHULTZ and LAMBERT (1973) NAGANO *et al.* (1983) DOREE *et al.* (1984)
— cyclic AMP	DOREE *et al.* (1981) MAZZEI *et al.* (1981) NEMOTO and ISHIDA (1983)
— cyclic GMP	NEMOTO and ISHIDA (1983)
— polyA-RNA	JEFFERY (1977)
— RNA	KOVESDI and SMITH (1982)

8. FERTILIZATION

8.1. Fertilization

Two precautions help in obtaining successful fertilization: addition of 10^{-4} M histidine increases sperm motility (Fujimori and Hirai, 1979) and calcium is required for acrosome reaction (Hagiwara and Dan, 1969).

In addition, since FOL (1879), it has been observed that successful fertlization can occur only at certain times during maturation: indeed during maturation the oocyte develops a cortical maturation (ability to elevate the fertilization membrane due to cortical exocytosis), a cytoplasmic maturation (ability to support sperm and chromatin decondensation and aster formation) and a nuclear maturation (formation of a haploid female pronucleus ready to fuse with the male pronucleus) and the possibility to develop a polyspermy block. For all these reasons, and although sperm can enter the oocyte at any moment before or during maturation, fertilization is optimal (monospermy and maximal regular cleavage) when insemination is performed between GVBD and the formation of the first polar body (Fujimori and Hirai, 1979; Hirai *et al.,* 1981; Schuetz and Longo, 1981; Longo and Schuetz, 1982).

A method has been described to separate sperm agglutinin and the acrosome reaction-inducing substance found in the egg jelly (Uno and Hoshi, 1978).

8.2. Parthenogenetic activation

Since the early work of Delage, Dalcq and Lillie (Delage, 1901; Delage and Goldsmith, 1922), using a pretreatment with carbonic acid-saturated water or high calcium concentrations ASW, or momentary elevation of the temperature, the starfish eggs have been widely used for the study of parthogenetic activation. As for fertilization the response of the oocyte varies throughout the maturation process. Although cortical reaction can be induced by ionophore A 23187 at any time, provided that the oocytes have been pretreated with CaFASW (Cayer *et al.,* 1975; Schuetz, 1975), other parthogenetic agents must be applied at specific times to induce full development. The most recently used agents are 1-MeAde itself °hich triggers complete activation and development when added after emission of the second polar body and formation of the pronucleus (Picard and Doree, 1982, 1983) and methylxanthines (6-10 mM) when added before formation of the second polar body (Obata and Nemoto, 1984).

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Berradenn

Atizan an darevin eus vigellouigoù Steredenn — Vor gant ur hormon naturel, ar 1-methyladenin zo deuet da vezañ ur skouer vat evit studiañ difreadur ar gellig gant obererien-diavaez. Taolennañ a ra ar pennad-mañ an oll teknikoù

implijet evit dielfennañ vigellig ar. Steredenn-Vor, en ur ziskouez pegen talvoudus eo an dañvez bevoniel-se. Dispiegañ a ra penaos prientiñ ispilhadennoù-kellouigoù unvan. Termeniñ a ra ar c'hiteria a zarevder hag ivez spisverkoù pouezhussañ an dareviñ : sinetik, live izellañ ar 1-methyladenin ha prantad hormon-ret. Diskrivañ a ra an teknikoù mikroensinklañ er c'hitoplasm hag en nukleüs hag ivez an arnodennoù a bep seurt a c'heller seveniñ dre implijoud vigellig ar Steredenn-Vor (tennan ar goc'henn gitoplasmek kuit, daougenteuziñ ar gelligoù, fardañ tammou nukleet pe dinukleet, lechliañ gorre ar vigellig, digengreizan ar vigelligad). Dispiega:ñi a ra penaos digenvezañ kortex, nuklei, gwerzhidadoù-meiozis ha lod a volekulennoù. Menegañ a ra erfin penaos sperian ha gwerc'hsperian ar vigellig.

Summary

Starfish oocyte maturation, induced bv the natural hormone 1-Methyladenine, has become a model for the study of cell activation by a specific and external agonist. This article reviews the different original techniques used in the study of this cell and points out the peculiar advantages of this marine biological material. The preparation of homogenous oocyte suspensions is decribed; the criterions of maturation are defined as well as its three major characteristics: 1-Methyladenine threshold concentration, kinetics of maturation and hormone-dependent period. The techniques of intracytoplasmic and intranuclear microinjections are shown in detail as well as the various possible manipulations of the oocytes (removal of the vitelline envelope, stratification of the oocyte, preparation of nucleated and anucleated fragments, oocyte fusions, local staining of the oocyte surface). The techniques of isolation of cortices, germinal vesicles, meiotic spindles and specific molecules are described. Finally, the conditions of fertilization and of parthenogenetic activation are presented.

REFERENCES

BENNETT, J. and MAZIA, п., 1981. — Interspecific fusion of sea urchin eggs. Surface events and cytoplasmic mixing. *Exp. Cell Res.,* 131, *pp.* 197-207.

BRYAN, J. and SATO, H., 1970. — The isolation of the meiosis in spindle from the mature oocyte of *Pisaster ochraceus. Exp. Cell Res.,* 59, *pp.* 371-378.

CARESTIA, c, GAUTHIER, s., GRANIER, A. and scARANO, E., 1977. — Purification and properties of a 3'-phosphonyl former endo deoxyribonuclease from egg of *Asteria forbesi. Biochemistry,* 16, *pp.* 3343-3347.

CAYER, MX., KISHIMOTO, т. and KANATANI, H., 1975. — Formation of the fertilization membrane by insemination of immature starfish oocytes pretreated with calcium-free seawater. *Dev. Growth and Different.,* 17, *pp.* 119-125.

DELAGE, v., 1901. — Etudes expérimentales sur la maturation cytologique et sur la parthénogenèse artificielle chez les Echinodermes. *Arch. Zool. Exp.,* 3, *pp.* 285-326.

DELAGE, Y., 1907. — Les vrais facteurs de la parthénogenèse expérimentale. *Arch. Zool. Exp. Gén.,* 7, *pp.* 445-506.

DELAGE, γ. et GOLDSMITH, н., 1922. — La parthénogenèse naturelle et expérimentale. Flammarion Edt., 344 *p.*

DOREE, M., 1980.— Calmodulin content does not change following hormone-induced meiosis reinitiation in starfish oocytes. *Experientia,* 36, *pp.* 932-933.

DOREE, M., 1981. — 1-Methyladenine induced stimulation of protein phosphorylation and Na^+ pump does not require the presence of the nucleus. *J. Exp. Zool.,* 217, *pp.* 147-150.

noREE, M.,MOREAU, M. and GUERRIER, p., 1978. — Hormonal control of meiosis. *In vitro* induced release of calcium ions from the plasma membrane in starfish oocytes. *Exp. Cell. Res.,* 115, *pp.* 251-260.

DOREE, M., PEAUCELLIER, G. and PICARD, A., 1983. — Activity of the maturation-promoting factor and the extent of protein phosphorylation oscillate simultaneously during meiotic maturation of starfish oocvtes. *Dev. Biol.,* 99, *pp.* 489-501,

FOL, H., 1879. — Recherches sur la fécondation et le commencement de l'embryogenie chez divers animaux. *Genève Soc. Phys. Mem.,* 26, pp. 89-397.

FUJIMORI, T. and HIRAI, S., 1979. — Differences in starfish oocyte susceptibility to polyspermy during the course of maturation. *Biol. Bull.,* 157, *pp.* 249-257.

FUSELER, J.W., 1973. — Repetitive procurement of mature gametes from individual sea stars. *J. Cell. Biol.,* 57, *pp.* 879-881.

GUERRIER, P., 1972. — Technique générale pour l'extraction du système périphérique total de l'ovocyte d'oursin. *Cah. Biol. Mar.,* 13, *pp.* 475-477.

GUERRIER, p., MEIJER, L., MOREAU, M. and LONGO, F.J., 1983. — Hormone-dependent GVBD induces cytoplasmic maturitv in the starfish oocyte. *J. Exp. Zool.,* 226, *pp.* 303-309.

HAGIWARA, Y. and DAN, J.C., 1969. — Effect of lack of calcium on the starfish acrosome. *Dev. Growth Differ.,* 11, *pp.* 29-39.

HARVEY, E.N., 1931. — The tension at the surface of marine eggs, especially those of the sea urchin *Arbacia. Biol. Bull.,* 61, *pp.* 273-279.

HARVEY, E.B., 1956. — The american *Arbacia* and other sea urchins. Princeton University Press.

HARAGUCHI, R. and NAGANO, H., 1983. — Isolation and characterization of DNA polymerases from mature oocytes of the starfish, *Asterina pectinifera. J. Biochem.,* 93, *pp.* 687-697.

HIRAI, s., KUBOTA, J. and KANATANI, H., 1971. — Induction of cytoplasmic maturation by 1-Methyladenine in starfish oocytes after removal of the germinal vesicle. *Exp. Cell. Res.,* 68, *pp.* 137-143.

HIRAMOTO, Y., 1974. — A method of microinjection. *Exp. Cell. Res.,* 87, *pp.* 403-406.

JEFFERY, W.R., 1977. — Polyadenylation of maternal and newly-synthesized UNA during starfish oocyte maturation. *Dev. Biol.,* 57, *pp.* 98-108.

KANATANI, H., SHIRAI, H., NAKANISHI, K. and KUROKAWA, T., 1969. — Isolation and identification of meiosis inducing substance in starfish *Asterias amurensis. Nature,* 221, *pp.* 273-274.

KANE, R.E., 1965. — The mitotic apparatus. Phvsical chemical factors controlling stability. *J. Cell. Biol.,* 25, *pp.* 137-144.

KISHIMOTO, T. and KANATANI, H., 1973. — Induction of starfish oocyte maturation by disulfide-reducing agents. *Exp. Cell Res.,* 82, *pp.* 296-302.

KISHIMOTO, T. and KANATANI, H., 1976. — Cytoplasmic factor responsible for germinal vesicle breakdown and meiotic maturation in starfish oocyte. *Nature,* 260, *pp.* 321-322.

KISHIMOTO, T. and KANATANI, H., 1977. — Lack of species specificity of starfish maturation - promoting factor. *Gen. Comp. Endocrinol.,* 33, *pp.* 41-44.

KISHIMOTO, T., KUBOTA, J. and KANATANI, H., 1977. — Distribution of maturation-promoting factor in starfish oocyte stratified by centrifugation. *Deo. Growth and Different.,* 19, *pp.* 283-288.

KISHIMOTO, T., KURIYAMA, R., HONDO, H. and KANATANI, H., 1982. — Generality of the action of various maturation-promoting factors. *Exp. Cell. Res.,* 137, *pp.* 121-126.

KOVESDI, I. and SMITH, M.J., 1982. — Sequence complexity in the maternal RNA of the starfish *Pisaster ochraceus* (Brandt). *Dev. Biol.,* 89, *pp.* 56-63.

LONGO, F.J. and SCHUETZ, A.W., 1982. — Male pronuclear development in starfish oocytes treated with 1-Methyladenine. *Biol. Bull.,* 163, *pp.* 453-464.

MABUCHI, I., 1974. — A myosin-like protein in the cortical layer of cleaving starfish eggs. *J. Biochem.,* 76, *pp.* 47-55.

MABUCHI, I., 1976. — Myosin from starfish eggs : properties and interaction with actin. *J. Mol. Biol.,* 100, pp. 569-582.

MARTINAGE, A., BELAICHE, D., DUPRESSOIR, T. and SAUTIERE, P., 1983. — Primary structure of histone H_2A from gonads of the starfish *Asterias rubens. Eur. J. Biochem.,* 130, pp. 465-472.

MARTINAGE, A., BRIAND, G., VAN DORSSELAER, A., TURNER, C.H. and SAUTIERE, P., 1985. — Primary structure of histone H2B from gonads of the starfish *Asterias rubens.* Identification of an N-dimethylproline residue at the amino-terminal. Eur. J. Biochem., 147, *pp.* 351-359.

MAZIA, D., MITCHISON, J.M., MEDINA, H. and HARRIS, P., 1961. — The direct isolation of the mitotic apparatus. *J. Biophys. Biochem., Cytol.,* 10, *pp.* 467-474.

MAZZEI, G., MEIJER, L., MOREAU, M. and GUERRIER, p., 1981. — Role of calcium and cyclic nucleotides during meiosis reinitiation. *Cell. Differ.,* 10, 139-145.

MEIJER, L. and GUERRIER, P., 1981. — Calmodulin in starfish oocytes. I. Calmodulin antagonists inhibit oocyte maturation. *Dev. Biol.,* 88, *pp.* 318-324.

MEIJER, L. and GUERRIER, p., 1983. — Immobilized methylglyoxal-bis(guanylhydrazone) induces starfish oocyte maturation. *Dev. Biol.,* 100, *pp.* 308-317.

MEIJER, L. and GUERRIER, P., 1984. — Maturation and fertilization in **starfish** oocytes. *Int. Rev. Cytol.,* 86, *pp.* 129-196.

MEIJER, L., GUERRIER, p. and MACLOUF, J., 1984. — Arachidonic acid, 12- and 15-hydroxyeicosatetraenoic acid, eicosapentaenoic acid and phospholipase **A,** induce starfish oocyte maturation. ***Dev. Biol.,*** 106, ***pp.*** 368-378.

MEIJER, L., MACLOUF, J. and BRYANT, R.W., 1985. — Arachidonic acid metabolism in starfish oocytes. ***Dev. Biol.,*** in press.

MEIJER, L. and WALLACE, R.W., 1980. — Calmodulin in starfish oocytes. ***Proc. Eur. Colloq. Echinoderms,*** Brussels, p. 385.

MEIJER, L. and WALLACE, R.W., 1985. — The role of calmodulin in oocyte maturation. In «Calmodulin antagonists and cellular physiology» (Hidaka, ed.), Academic Press, ***pp.*** 129-145.

MITA, L. and OBATA, c, 1984. — Timing of early morphogenetic events in tetraploid starfish embryos. *J. **Exp. Zool.,*** 229, ***pp.*** 215-222.

NAGANO, H., OKANO, K. and IKEGAMI, s., 1983. — Changes in deoxyribonucleoside triphosphate pools in the starfish oocyte during maturation and early embryogenesis. *Exp. Cell Res.,* 145, *pp.* 219-222.

NEMOTO, S.I. and ISHIDA, I., 1983. — Changes in cGMP levels on meiosis reinitiation **of** starfish oocytes. ***Exp. Cell Res.,*** 145, ***pp.*** **226-230.**

NEMOTO, S.I., YONEDA, M. and **UEMURA, I., 1980. —** Marked decrease in the rigidity **of** starfish oocytes induced **by** 1-Methyladenine. ***Dev. Growth and Different.,*** 22, ***pp.*** **315-325.**

OBATA, c. and **NEMOTO, S.I., 1984. —** Artificial parthenogenesis in starfish eggs **:** production **of** parthenogenetic development through suppression **of** polar body formation **by** methylxanthines. ***Biol. Bull.,*** 166, **525-536.**

OISHI, N. and **SHIMADA, H., 1983. —** Intracellular localization **of DNA** polymerases in **the** oocyte **of** starfish, ***Asterina pectinifera. Dev. Growth and Different.,*** **25,** ***pp.*** **547-551.**

OISHI, N. and **SHIMADA, H., 1984. —** Intracellular localization and sedimentation coefficient **of** DNA ligase in oocytes **of** the starfish, ***Asterina pectinifera.*** Dev. **Growth** and Different. 26, **pp. 571-574.**

PICARD, A. et **DOREE, M., 1982. — La** 1-Methvladenine provoque l'activation **parthénogénétique** des ovocytes d'étoile de mer. ***C.R. Acad. Sci., Paris,*** 295, ***pp.*** **311-314.**

PICARD, A. and **DOREE, M., 1983. —** Hormone-induced parthenogenetic activation **of** mature starfish oocytes. ***Exp. Cell. Res.,*** **145,** ***pp.*** **315-323.**

RICHTER, H.p., SCHEURICH, p. and **ZIMMERMAN, u., 1981. —** Electric field-induced fusion **of** sea urchin eggs. ***Develop. Growth and Differ.,*** 23, ***pp.*** **479-486.**

ROTSCHILD, L., 1950. — Counting spermatozoa. ***J. Exp. Biol.,*** **26,** ***pp.*** **388-395.**

SAKAI, H., 1968. — Contractile properties **of** protein threads **from** sea urchin eggs **in** relation **to** cell division. ***Int. Rev. Cytol.,*** 23, ***pp.*** **89-112.**

SCHROEDER, T.E. and **STRICKER, S.A., 1983. —** Morphological changes during maturation **of** starfish oocytes **:** surface ultrastructure and cortical actin. ***Dev. Biol.,*** 98, ***pp.*** **373-384.**

SCHUETZ, A.w., 1975. — Cytoplasmic activation **of** starfish oocytes **by** sperm and divalent ionophore **(A 23187). *J. Cell. Biol.,*** **66,** ***pp.*** **86-94.**

SCHUETZ, A.W. and LONGO, **P.J., 1981. —** Hormone-cytoplasmic interactions controlling sperm nuclear decondensation and male pronuclear development in starfish oocytes. *J. **Exp. Zool.,*** **215,** ***pp.*** **107-111.**

SCHULTZ, T.w. and LAMBERT, C.C., **1973. —** Changes in adenine nucleotide levels and respiration during 1-Methyladenine induced maturation **of** starfish oocytes. ***Exp. Cell Res.,*** 81, ***pp.*** **163-168.**

SEKIRINA, G.G., SKOBLINA, **M.N., VASSETZKY, S.G.** and **BILINKIS, A.A., 1983.** — The **fusion** **of** oocytes **of** the starfish ***Aphelasterias japonica.*** **I.** Formation **of** cell hybrids. ***Cell. Different,*** **12,** ***pp.*** **67-71.**

SHAPIRO, H., 1941. — Centrifugal elongation **of** cells and **some** conditions governing **the** return **to** sphericity **and** cleavage **time.** ***J. Cell Comp. Physiol.,*** 18, ***pp.*** **61-78.**

SHIDA, H. and **HIRAI, s., 1978. —** Site **of** 1-Methyladenine receptors in the maturation **of** starfish oocytes. ***Dev. Growth and Differ.,*** **20,** ***pp.*** **205-211.**

SHIRAI, H. and KANATANI, H., 1980. — Effect of local application of 1-Methyladenine on the site of polar body formation in starfish oocyte. ***Dev. Growth and Different.,*** **22, pp. 555-560.**

SHIRAI, H. and KANATANI, H., 1982. — Effect of 1-Methyladenine on responses of spermatozoa to egg jelly in starfish — a convenient method for counting the rate of acrosome reaction and for measuring sperm motility. *Zool. Mag.*, 91, pp. 272-280.

THALLER, MM, cox, M.C.L. and VILLEE, C.A., 1969. — Isolation of nuclei from sea urchin eggs and embryos. *J. Cell. Biol,* 57, *pp.* 846-850.

UNO, Y. and HOSHI, M., 1978. — Separation of the sperm agglutinin and the acrosome reaction-inducing substance in egg jelly of starfish. *Science,* 200, *pp.* 58-59.

VACQUIER, v. and PAYNE, J.E. (1973). Methods for quantitating sea urchin sperm-egg binding. *Exp. Cell Res.*, 82, *pp.* 227-235.

VANHOUTTE-DURAND, **G.,** MIZON, J., SAUTIERE, P. and BISERTE, **G.,** 1977. — Histones from gonads of the starfish *Asterias rubens. Comp. Biochem. Physiol.*, 57B, *pp.* 121-126.

VASSETZKY, S.G., SEKIRINA, **G.G.,** SKOBLINA, M.N. and BILINKIS, A.A., 1983. — The fusion of oocytes of the starfish *Aphelasterias japonica.* II. The capacity of cell hybrids for maturation and cleavage. *Cell. Different.,* 12, *pp.* 73-76.

VASSETZKY, **S.G.,** SEKIRINA, G.G., VEISMAN, B.L., SKOBLINA, M.N. and BILINKIS, A.A., 1984. — The fusion of oocytes of the starfish *Aphelasterias japonica.* II. Reconstruction of oocytes from cells and cell fragments (cytoplasts). *Cell Different.,* 14, *pp.* 47-52.

VOOGT, P.A. and VAN RHEENEN, J.W.A., 1979. — Studies on the possible regulatory function of asterosaponins in oocyte maturation and early embryogenesis of *Asterias rubens. Int. J. Invert. Reprod.*, 1, *pp.* 307-316.

YAMAMOTO, K. and YONEDA, M., 1983. — Cytoplasmic cycle in meiotic division of starfish oocytes. *Deo. Biol.*, 96, *pp.* '166-172.

ZIMMERMAN, U., 1982. — Electric field-mediated fusion and related electrical phenomena. *Bioch. Biophys. Acta,* 694, *pp.* 227-277.